PRODUCTION OF EXTRACELLULAR PROTEASE ENZYME BY *ASPERGILLUS NIGER*

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ABSTRACT

The research aimed to study the production of extracellular protease enzyme using potato peel extract as the additional carbon source for *Aspergillus niger*. Proteases are catalytically functioned to hydrolyze or breakdown the peptide bonds of proteins. Proteases are found to be used in many biotechnological processes and industrial applications such as in baking industry for gluten development, dairy industry as milk-clotting agents and pharmaceutical industries. Wastes from the agricultural and food industry gives out serious problem and as an action of initiative, those wastes can be used up and converted into value added materials as well as cost-effective substrates for fermentation of extracellular protease enzyme. Furthermore, in order to produce high yields of protease enzymes, the optimization of parameters is considered vital since it takes a long time and expensive to be optimized conventionally. This study was performed by using Response Surface Methodology (Central Composite Design). *Aspergillus niger* had been chosen as biomass while potato peel from the agricultural and food industry was used as additional substrate in this study. The potato peel will be grinded and blended with peel:water ratio of 1:3. The fermentation study will be take place in the shake flasks and several parameters were optimized for higher protease enzyme activity. Three factors were taken into considerations which were the pH of the fermentation medium (pH 3.5 – pH 7.5), the substrate concentration (20 g/l – 60 g/l) and the agitation speed (100 rpm – 300 rpm). From OFAT analysis, protease enzyme showed the optimum activity at pH 5.50, 40 g/l and 200 rpm with 1.23 U/ml, 1.57 U/ml and 1.38 U/ml, respectively while RSM results depicted that the optimum values of each parameter were 5.5 for pH, 40 g/l of substrates concentration and 200 rpm of agitation speed which gave out the optimum protease activity of 2.4563 U/ml. As the conclusion, RSM is the best tool used to identify the correlation between controlled independent factors and observed dependent responses and the utilization of waste as the fermentation substrates is highly acceptable due to its higher protease activity. For future study, it is recommended for an optimization of potato peel extracts concentration as the main carbon source, the application of genetic engineering in the enzyme production, further scale up protease production using a bioreactor and purification and toxicology studies on protease enzyme for further used by human, food and pharmaceutical industries.

ABSTRAK

Penyelidikan ini bertujuan untuk mengkaji penghasilan enzim protease di luar sel menggunakan ekstrak kulit kentang sebagai sumber karbon tambahan untuk *Aspergillus niger*. Protease berfungsi untuk menghidrolisis dan memutuskan ikatan-ikatan peptida yang terdapat pada protein. Ia banyak digunakan dalam proses-proses bioteknologi dan diaplikasikan dalam pelbagai industri seperti industri penaik sebagai peningkat gluten, industri tenusu sebagai agen penggumpal susu dan industri farmaseutikal. Peningkatan bahan buangan yang terhasil daripada industri pertanian dan industri makanan menjadi satu masalah besar dan sebagai langkah inisiatif, bahan-bahan buangan tersebut boleh digunakan dan ditukar menjadi sesuatu yang bernilai sebagai substratu berkos rendah dalam proses fermentasi untuk penghasilan enzim protease. Tambahan pula, pengoptimuman parameter-parameter tertentu sangat penting untuk meningkatkan penghasilan enzim protease. Pengoptimuman secara konvensional mengambil masa yang lama dan memerlukan kos yang tinggi. Kajian ini dijalankan dengan menggunakan Kaedah Tindak Balas Permukaan (Reka Bentuk Komposit Berpusat). *Aspergillus niger* telah dipilih sebagai biomas manakala kulit kentang dari industri pertanian dan industri makanan telah digunakan sebagai substratu dalam kajian ini. Kulit kentang dikisar dan dicampur dengan nisbah kulit kentang (1) kepada air (3). Kajian fermentasi ini dilakukan dalam kelalang goncang dan sesetengah parameter telah dikaji untuk penghasilan enzim protease yang optimum. Tiga parameter telah dikaji iaitu pH media fermentasi (pH 3.5 – pH 7.5), kepekatan substratu (20 g/l – 60 g/l) dan kelajuan pengadukan (100 rpm – 300 rpm). Merujuk kepada analisa OFAT, enzim protease menunjukkan aktiviti optimumnya berlaku pada pH 5.50, 40 g/l dan 200 rpm dengan 1.23 U/ml, 1.57 U/ml dan 1.38 U/ml, masing-masing manakala keputusan RSM menunjukkan nilai optimum untuk setiap parameter ialah pH 5.50, kepekatan substratu pada 40 g/l dan kelajuan pengadukan pada 200 rpm dengan aktiviti protease optimumnya pada 2.4563 U/ml. Kesimpulannya, RSM merupakan alat yang terbaik untuk mengenalpasti kaitan antara faktor bebas yang boleh dikawal dengan faktor bergantung yang diperhatikan dan penggunaan bahan buangan sebagai substratu bagi proses fermentasi adalah sangat digalakkan memandangkan lebih banyak enzim protease yang dihasilkan. Untuk kajian masa depan, adalah digalakkan untuk mengoptimumkan kepekatan ekstrak kulit kentang yang digunakan sebagai sumber karbon utama, penggunaan kejuruteraan genetik dalam penghasilan enzim, meningkatkan skala penghasilan enzim pada masa akan datang dengan menggunakan bioreaktor dan kajian tentang penulenan dan toksikologi enzim protease yang bakal digunakan oleh manusia, industri makanan dan farmaseutikal.

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

Protease is an enzyme that hydrolyzed peptide bonds (Salahuddin and Khan, 2008) and their usage in many industries is so much impressive. In dairy industry, protease is being used to coagulate the milk protein forming curds and ready to be used for cheese preparation. In food industry, proteases were used for improving the functional, nutritional and flavour properties in proteins especially in baking where it is used to degrade proteins in flour for biscuits, crackers and cookies. In pharmaceutical industry also, protease give a wide application such as in treatment of clotting disorder (Sumantha *et al.*, 2006). It has been used for treatment of clotting disorder in pharmaceutical industry while in detergent industry, used for protein stain removal. For leather industry, it usage is for unhearing and bating. According to Oyeleke *et al.* (2010), protease can easily isolate from various sources as well as plants, animals and microbial via fermentation process.

Enzyme production is a good value added to agro-industrial residues since they can be used in the production of enzymes by bioprocesses (Paranthaman *et al.*, 2009). This issue was related to **"**From waste to wealth" concept which is an idea that had been practiced nowadays by almost all people in the world. This statement gives an idea of using unwanted materials that can be recycled or becoming alternatives resources for other processes. The unwanted materials will basically refer to the wastes. An example of wastes come from agricultural and food industry because there are most abundant of agro-industrial residues on our Earth that actually possess such good potential as renewable

resources (Nigam and Pandey, 2009). The utilization of wastes as substrate in the industrial enzyme production has made the fermentation process of industrial enzymes economically feasible.

The aim of this study is to investigate the production of extracellular protease enzymes by *Aspergillus niger* using potato peel extracts as substrate and to optimize several fermentation parameters for optimum enzyme activity.

1.2 PROBLEM STATEMENT

Many researches has been done for developing processes to produce extracellular protease enzyme that involves various additional process for improvement in terms of economical, qualitative, quantitative, and process performance factors. The screening of microorganism, the preparation of the substrate from various sources and the selection of range of several parameters are examples of improved process being done.

Around 40% for the whole cost of production is the substrates cost (El Enshasy *et al.*, 2008). Thus, the availability of an inexpensive raw material; potato peel extracts which will be used in this research is essential if this fermentation is to become economically viable.

In fermentation, there is variety of parameters that could affect the process performance. Fermentation is known to be dependent on temperature, pH, rate of aeration, substrate concentration etc so, that is why optimization steps need to be done in order to maximize the production rate of protease enzymes (El Enshasy *et al.*, 2008). The parameters need to be controlled to provide the perfect fermentation conditions for the fermentation process.

1.3 OBJECTIVE

The objective of this research is to study the production of extracellular protease enzyme using potato peel extracts as the additional substrates for *Aspergillus niger*.

1.4 SCOPE OF THE STUDY

To achieve the objective, few scopes have been identified in this research:

a) To study the production of extracellular protease enzyme in submerged fermentation using potato peel extracts as the additional substrate for *Aspergillus niger*.
b) To study the effects of pH (pH 3.5, 4.5, 5.5, 6.5 and 7.5) to the extracellular protease production.
c) To study the effects of substrate concentration (20, 30, 40, 50 and 60 g/l) to the extracellular protease production.
d) To study the effects of agitation speed (100, 150, 200, 250 and 300 rpm) to the extracellular protease production.
e) To apply Response Surface Methodology (RSM) in designing the experimental work.

1.5 RATIONAL AND SIGNIFICANCE

The aim of this study was to explore for new beneficial sources of substrate from wastes of agricultural and food industry so that the cost of growth factors can be lower down. This study proposed in minimizing the waste problems in the industry and estimating the optimum parameters values for higher yield of extracellular protease production by using potato peel extracts as the substrate to *Aspergillus niger*. The application of Response Surface Methodology (RSM) was a step of development in today's technology and this study was to expose others with new born technology. Perhaps with the application of RSM, experimental works will be much easier, less time consuming and feasibly inexpensive. The experimental stages done and obtained from the present study may be review by other future researchers for further process development in this field.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

This part included all the collected and well organised information related to the study from variety of literature being reviewed from journals and other related sources.

2.2 INDUSTRIAL ENZYMES

Smythe (1950) defined enzymes as organic catalysts which are produced by living cells and they are responsible in catalyzing the chemical reactions of process life of the cell. There are many different kinds of enzymes and their existence is actually undetermined since only few known enzymes had been discovered. Some people proposed that all proteins are enzymes.

Global Industry Analyst, Inc. (GIA) forecasting the global market for industrial enzymes by 2015 is approximately to be US$3.74 billion. This is due to new developed enzyme technologies that enhance the cost efficiencies and productivity, customers' favour in substituting petrochemical products with organic products and lastly because of the high demand from textile, detergent, cosmetic and pharmaceuticals manufacturers (Global Industry Analysts, 2011). The common enzymes are lipases, carbohydrases, proteases and many more. The role of industrial enzyme had also been studied by Kirk *et al*. (2002) and is showed in the Table 2.1.

Table 2.1: Role of industrial enzymes

Industry	Enzyme class	Application
Detergent (laundry and dish washing)	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipase	Lipid stain removal
	Cellulase	Cleaning, colour clarification, anti-redeposition (cotton)
	Mannonase	Mannanan stain removal (reappearing stains)
Starch and fuel	Amylase	Starch liquefaction and saccharification
	Amyloglucosidase	Saccharification
	Pullulanase	Saccharification
	Glucose isomerise	Glucose to fructose conversion
	Xylanase	Viscosity reduction (fuel and starch)
	Protease	Protease (yeast nutrition – fuel)
Food (including dairy)	Protease	Milk clotting, infant formulas (low allergenic), flavour
	Lipase	Cheese flavour
	Lactase	Lactose removal (milk)
	Pectin methyl esterase	Firming fruit-based products
	Pectinase	Fruit-based products
	Transglutaminase	Modify visco-elastic properties
Baking	Amylase	Bread softness and volume, flour adjustment
	Xylanase	Dough conditioning
	Lipase	Dough stability and conditioning (*in situ* emulsifier)
	Phospholipase	Dough stability and conditioning (*in situ* emulsifier)
	Glucose oxidase	Dough strengthening
	Lipoxygenase	Dough strengthening, bread whitening
	Protease	Biscuits, cookies
	Transglutaminase	Laminated dough strengths

Table 2.1: Continued

Industry	Enzyme class	Application
Animal feed	Phytase	Phytate digestibility – phosphorus release
	Xylanase	Digestibility
	β-glucanase	Digestibility
Beverage	Pectinase	De-pectinization, mashing
	Amylase	Juice treatment, low calorie beer
	β-glucanase	Mashing
	Laccase	Clarification (juice), flavour (beer)
Textile	Cellulase	Denim finishing, cotton softening
	Amylase	De-sizing
	Pectate lyase	Scouring
	Catalase	Bleach termination
	Laccase	Bleaching
	Peroxidase	Excess dye removal
Pulp and paper	Lipase	Pitch control, contaminant control
	Protease	Biofilm removal
	Amylase	Starch-coating, de-inking, drainage improvement
	Xylanase	Bleach boosting
	Cellulase	De-inking, drainage improvement, fiber modification
Fats and oils	Lipase	Transesterification
	Phospholipase	De-gumming, lyso-lecithin production
Organic synthesis	Lipase	Resolution of chiral alcohols and amides
	Acylase	Synthesis of semisynthetic penicillin
	Nitrilase	Synthesis of enantiopure carboxylic acids
Leather	Protease	Unhearing, bating
	Lipase	De-pickling

Source: Kirk *et al.* (2002)

One of the largest product segments in global industrial enzyme market is proteases (Rao *et al*., 1998; Global Industry Analysts, 2011). Proteases monopolized around 60% of the world's industrial enzyme market as they are found to be used in many biotechnological processes and industrial applications (Rao *et al*., 1998; El Enshasy *et al.*, 2008) such as in baking industry for gluten development, dairy industry as milk-clotting agents (Sumantha *et al.*, 2006) and pharmaceutical industries (Sambamurthy and Kar, 2006) and this is clearly seen in Figure 2.1.

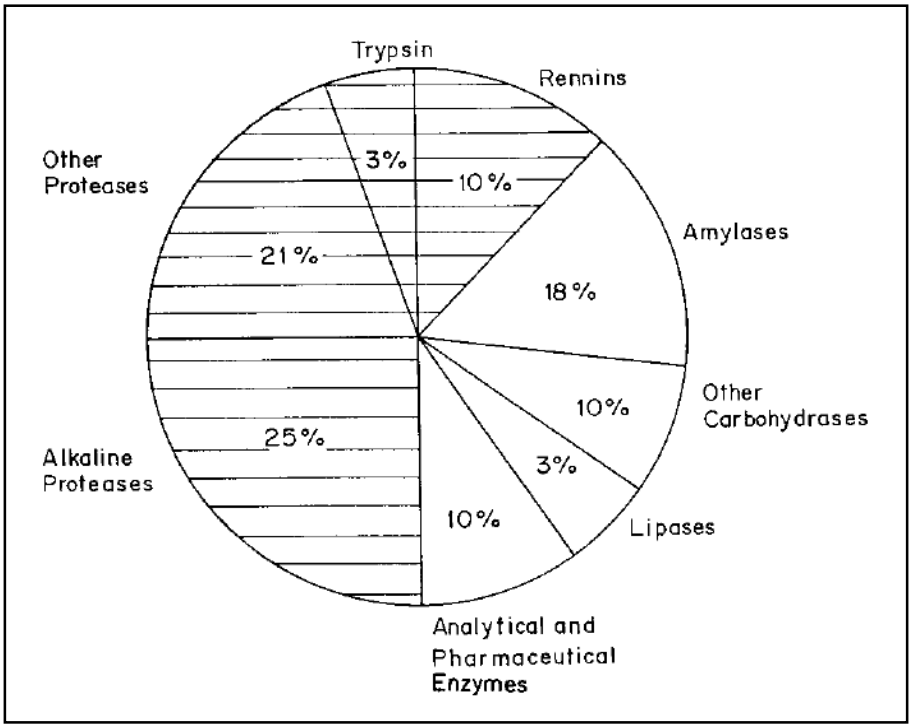

Figure 2.1: Distribution of enzymes sale

Source: Rao *et al*., 1998.

The Figure 2.1 describes the sale distribution of enzymes. The shaded area represents the sale of proteases enzyme and it shows that half of the sale is monopolized by protease. This figure really proved that proteases are important enzyme used in the world.

Since proteases were found to have such numerous applications in the industries, they were commercially produced nowadays.

2.3 PROTEASE

Proteases, also known as proteolytic enzymes or proteinases, belong to a group of enzymes whose catalytically function to hydrolyze or breakdown the peptide bonds of proteins and they can either be limited proteolysis which break specific peptide bonds or unlimited proteolysis which break down a complete polypeptide chain to amino chain residues (Salahuddin and Khan, 2008).

These enzymes can be found from various sources such as plants, animals and microorganisms (Rao *et al*., 1998; Ikram-Ul-Haq *et al.*, 2006). Protease can be classified according to three major criteria which are the type of reaction catalyst, chemical nature of the catalytic site and evolutionary relationship with reference to structure. Rao *et al.* (1998) studied further about microbial proteases. There are two major fungi that responsible in the production of protease which is filamentous fungi and yeast and can be further classified to acidic protease, alkaline protease, serine protease and metalloprotease (filamentous fungi)/other protease (yeast).

Mostly they were produced by fungi, bacteria and viruses (microbial proteases) (Rao *et al*., 1998; Ikram-Ul-Haq *et al.*, 2006) because of the inability of plants and animals proteases to satisfy the world demands (Rao *et al*., 1998; Kumar *et al.*, 2008). Adinarayana and Ellaiah (2002) also reported that two third of industrial proteases are microbial proteases. This is due to their broad biochemical diversity and susceptibility to genetic manipulation (Sandhya *et al.*, 2005) and also because of the characteristics of microbial proteases that can satisfy the need in biotechnological application (Kumar *et al.*, 2008). Table 2.2 shows the potential producers of proteases according to its types (Rao *et al*., 1998).

Table 2.2: Fungal proteases producers

Fungi	Type of proteases	Fungi species
Filamentous fungi	Acid proteases	a) *Mucor* b) *Rhizopus* c) *Aspergillus*
	Alkaline proteases	a) *Aspergillus* b) *Acremonium* c) *Fusarium*
	Serine proteases	a) *Tritirachium*
	Metalloproteases	a) *Aspergillus*
Yeast	Acid proteases	a) *Saccharomycopsis* b) *Saccharomyces* c) *Candida albicans, Candida tropicalis* d) *Yarrowia lipolytica*
	Alkaline proteases	a) *Yarrowia lipolytica*
	Serine proteases	a) *Kluyveromyces* b) *Saccharomyces cerevisiae*
	Other proteases	a) *Saccharomyces cerevisiae*

Source: Rao *et al*. (1998)

Almost all industries have long used bacterial proteases but the preparation for obtaining enzymes that free from microbes is quite costly (Andrade *et al.*, 2002). Due to this issue, fungal proteases have increase in their industrial demand since they offer several advantages compared to bacterial proteases.

Sandhya *et al.* (2005) and Murthy and Naidu (2010) stated that fungi were safe in producing enzymes because they were known as GRAS (Generally Recognized As Safe) strains and referring to Food and Drug Administration (2001), proteases from *Aspergillus niger* and *Aspergillus oryzae* origins were declared to be GRAS, meaning that they are safe to be consumed by human. In addition, fungal protease was produced extracellular that make ease to be recovered from the fermentation broth (Sandhya *et al.*, 2005; Murthy and Naidu, 2010). Moreover, the generated mycelium from the fermentation system can be easily tossed by simple filtration (Andrade *et al.*, 2002; Murthy and Naidu, 2010) and fungi

can also easily grown on inexpensive substrates (Murthy and Naidu, 2010; Benazir *et al.*, 2011). Besides, these microorganisms are also capable in consuming organic materials in wastes for their both carbon and energy sources in order to grow (Mahmood *et al.*, 1998).

Proteases can be produced either intracellularly or extracellularly. Mostly microbial proteases were produced extracellularly which means the proteases abundantly found within the production medium. Andrade *et al.* (2002) said that extracellular enzymes capable to digest insoluble nutrient materials such as cellulose, protein and starch, and the nutrients from digested products are then transported into the cell to be used as for growth.

2.4 *ASPERGILLUS NIGER*

Aspergillus niger is one of the known fungi that have the potential in producing extracellular protease enzymes due to its cosmopolitan and ubiquitous nature (Benazir *et al.*, 2011). The scientific classification of *Aspergillus niger* is described by Table 2.3.

Table 2.3: Taxonomy of *Aspergillus niger*

Kingdom	Fungi
Phylum	Ascomycota
Class	Eurotiomycetes
Order	Eurotiales
Family	Trichocomaceae
Genus	*Aspergillus*
Species	*Aspergillus niger*

Source: (Universal Protein Resources, 2011)

Aspergillus niger is a filamentous fungus that give an important role in biotechnology. *Aspergillus niger* does have spores and reproduces asexually meaning that it can produce its offspring individually. Purwanto *et al.* (2009) discovered the morphology of *Aspergillus niger* using Scanning Electron Microscope (SEM) while Hoffmann (2010) identified the SEM of the asexual reproduction of *Aspergillus niger*.

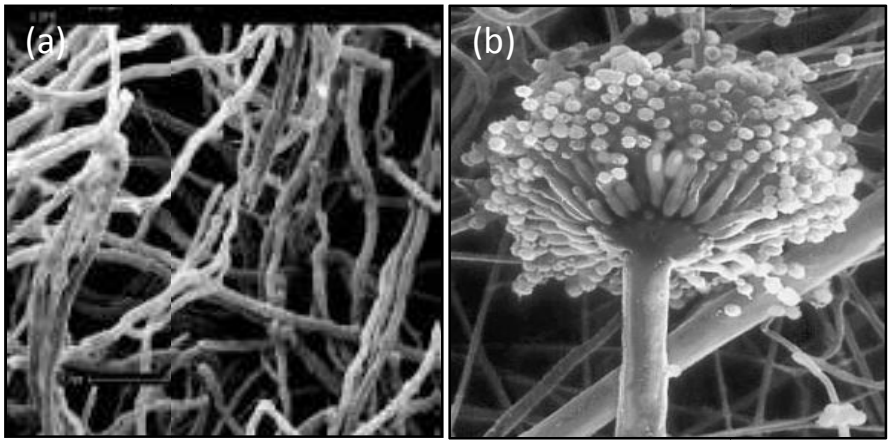

Figure 2.2: (a) Filamentous structure of *Aspergillus niger*
(b) Asexual reproduction of *Aspergillus niger*

Sources: (a) Purwanto *et al.* (2009)
(b) Hoffmann (2010)

According to Ikram-Ul-Haq *et al.* (2006), filamentous fungi do have potential to grow under varying environmental conditions such as fermentation time, pH, temperature and utilizing various sources of substrate as nutrients. Several fungi have been reported to produce proteases such as *Aspergillus niger* (Paranthaman *et al.*, 2009), *Aspergillus oryzae* (Murthy and Naidu, 2010), *Aspergillus fischeri* (Saravanakumar *et al.*, 2010), *Penicillium chrysogenum* (Ikram-Ul-Haq *et al.*, 2006) and *Mucor circinelloides* (Andrade *et al.*, 2002).

2.5 PRODUCTION OF PROTEASES

Microbial proteases have been commercially produced by fermentation process either in solid-state fermentation (SSF) or submerged fermentation (SmF). In large scale fermentation, SSF is not often used due to severe engineering problems and it was suggested to apply the submerged fermentation process as it possesses few advantages (Gregori *et al.*, 2007). Submerged fermentation was said to provide more uniform and reproducible biomass and also requires uncomplicated downstream processing.

Some researcher also discovered other method that was capable in producing high yield of protease enzymes. Samarntarn *et al.* (1999) used genetically engineered *Aspergillus oryzae* U1521 that enabled to produce five times more proteases yield than its parental strain since it contains multiple copies of protease gene while Dunne *et al.* (1997) had successfully forced *Stenotrophomonas maltophilia* W18 which was isolated from the rhizosphere of sugar beet to secrete extracellular protease activity for the purpose of biological control of *Pythium ultimum*. Unfortunately, the application of genetic engineered technique was still lacking among the researchers and most of them focused more in using the common fermentation technique either in submerged or solid state forms.

With regard to proteases synthesis in the microorganism, Saravanakumar *et al.* (2010) discovered that medium composition and some physical factors such as the pH, fermentation period, temperature etc have influenced the extracellular protease production. El Enshasy *et al.* (2008) decided that the improvement of yields of proteases and the optimization of the fermentation medium and production conditions need to be considered as to develop economically feasible technology. That was why many parameters were studied in former researches for examples, the pH, temperature, incubation time, different carbon and nitrogen sources, substrate concentrations and inoculums sizes. Better fermentation conditions will enhance higher production of proteases.

There will be many variables to be studied in every fermentation process. Studying one parameter at a time while holding the other parameters constant is the optimization

process that is done classically will normally takes such a long period of time and quite costly (Saravanakumar *et al.*, 2010). It also does not consider the effect of interaction of various parameters (Adinarayana and Ellaiah, 2002). Response Surface Methodology (RSM) is known as a useful model for studying factors that affect the responses by varying them simultaneously without much number of experiments to be carried out (Adinarayana and Ellaiah, 2002). With this, optimization procedure will be much easier to carry out.

2.5.1 Effect of pH on Protease Production

The pH is the measure of acidity or alkalinity of an aqueous solution and it has been proved strongly to affect the production of protease in the process of fermentation. It influences most of the enzymatic processes and the transport process of diverse of components across the cell membrane (Sandhya *et al.*, 2005; Paranthaman *et al.*, 2009; Murthy and Naidu, 2010).

The pH is related to the amount of hydrogen (H^+) concentration and this H^+ will give effect to the growth of the microorganisms. At neutral pH, it can act as the substrate that might be used by the cells in order to growth but in acidic and alkaline conditions, it can become inhibitor to the cell growth and this will affect the enzyme production by the cells. Natarajan and Rajendran (2009) also said that variety of pH values may also lead to the changes of protein structure meaning that maybe at certain pH the protease might be denatured once it is secreted to the outside of the cells or it can be said that the enzyme is already inactive and not stabile at that pH.

Murthy and Naidu (2010) found out that the increase in pH can cause the decrease of protease activity. In addition, different microorganisms might have different kinds of metabolic process and due to that the enzyme activity might also be affected. Coral *et al.* (2002) reported that fungal proteases are active at neutral pH and alkaline pH around pH 7 to pH 11 but Chakrabarthi *et al.* (2000) proved that *Aspergillus terreus* managed to produce protease between pH 5.5 and pH 9.5.

2.5.2 Effect of Substrate Concentration on Protease Production

All microorganisms need carbon sources in order to live as it is the food for them. According to Adinarayana *et al.* (2003), the carbon sources are the utmost nutritional factors that influenced the protease production.

Logically, if the microorganisms are supplied with adequate amount of nutrients, they will grow effectively meaning that they got all the nutrients needed in order to grow and product production will be maximized. If the microorganisms grow in lack of nutrients, their growth may be retarded and this will probably affect the product formation within the fermentation process.

Other metabolites or maybe the main products itself can repressed the product formation and it said that commonly the product production will decrease if the substrate concentration increase (Escobar and Barnett, 1993). When the substrate concentration is too high, it may also prohibit the enzyme activity (Dekleva *et al.*, 1985). Andrade *et al.* (2002) showed that protease activity was at its maximum when 40 g/l of substrate was used.

2.5.3 Effect of Agitation Speed on Protease Production

Agitation speed is one of the physical factors the influenced the fermentation process and at the same time give effect to the production of the product by the microorganisms. Several researchers did study the effect of agitation speed towards the protease production.

In the fermentation of aerobic culture, the oxygen really affects the production of product. It is because the metabolic pathway had been affected and the changes in metabolic fluxes occurred (Calik *et al.*, 1998). According to Ducros *et al.* (2009), the respiration rate of the aerobic culture is dependent to the dissolved oxygen if the dissolved oxygen reaches below its critical level. This is due to physiological alteration in cell metabolism (Hwang *et al.*, 1991). In order to avoid the reduce in cell growth and product

formation, it is necessary to make sure the agitation speed supplied for the fermentation is adequate enough since the agitation enhances the dispersion of air in the fermentation medium which makes it capable in maintaining the dissolved oxygen level (Ducros *et al.*, 2009; Singh *et al.*, 2011), equilibrate the temperature and the pH (Ducros *et al.*, 2009) and also improves nutrient transfer rate (Ducros *et al.*, 2009; Kamath *et al.*, 2010). As the matter of fact, high aeration rate causes by the agitation will improve enzyme synthesis.

Unfortunately, optimum enzyme production influences by diverse agitation ranges. Sepahy and Jabalameli (2011) suggested that agitation speed at 110 rpm and 130 rpm gave lower protease activity while some other researchers reported the optimum agitation speed for protease production from different isolates were at a range of 150 – 300 rpm (Banerjee *et al.*, 1999; Joo *et al.*, 2002; Kanekar *et al.*, 2002). Aerobic culture unable to grow in anaerobic condition so due to lack of aeration and nutrients uptake affected by the agitation, the cells cannot grow well but the same thing will also happened if the speed of agitation was too high. High speed will damage the microorganisms and its morphology due to shear stress and will also probably become the factor of low enzymatic activity (Ducros *et al.*, 2009; Sepahy and Jabalameli, 2011; Singh *et al.*, 2011).

2.6 UTILIZATION OF WASTES AS SUBSTRATE

Fermentation process needs nutrient medium that must satisfy the elemental requirements for the cell biomass, product formation and energy. The nutrients consist of macronutrients such as carbon sources, nitrogen sources and micronutrients such as trace elements and vitamins. According to El Enshasy *et al.* (2008), 40% of the production cost of large scale industrial enzyme is based on the cost of substrate so, it is important to use cost-effective substrate in the production process.

Normally most research used alternative sources of substrate to substitute the carbon and nitrogen sources in the fermentation process. There are many relevant sources of substrate to be used in the fermentation and the utilization of these alternative sources is hopefully promising for cheaper substrate cost but with efficient enzyme production especially in large scale production.

Agricultural, food and drinks industrial wastes are becoming abundant (Mahmood *et al.,* 1998) and the wastes disposal is problematic (Gregori *et al.,* 2007). Due to the pollution cause by this issue, Sarkar *et* al. (2011) had come out with biological treatment of these wastes via microbial degradation and it was the efficient method to be used in the future. By the way, these organic materials are composed of carbohydrate, amino acids, peptides and proteins, volatile acids, fatty acids and esters that are known to be biodegradable (Sarkar *et* al., 2011) while Mahmood *et al.* (1998) also discovered that the wastes of orange and potato peel extracts do have significant amount of carbohydrates except cellulose and they are readily to be used by the microorganisms while the microorganisms are said to be capable to utilize the organic matter in wastes as sources of either energy or carbon for their growth. Due to the above reasons, nowadays, the usage of agricultural, food and drinks wastes as fermentation substrates is highly increasing as it promotes lower cost requirement.

Recent works have studied a variety of wastes as carbon and nitrogen sources in the production of proteases such as orange and potato peel extracts (Mahmood *et al.,* 1998),

spent brewing grain, coconut oil cake, palm kernel cake, sesame oil cake, jackfruit seed powder and olive oil cake (Sandhya *et al*., 2005), rice husk (Sandhya *et al*., 2005; Ahmed *et al.*, 2010), rice bran (Sandhya *et al*., 2005; Ahmed *et al.*, 2010; Benazir *et al*., 2011), wheat bran (Sandhya *et al*., 2005; Kumar *et al*., 2008; Ahmed *et al.*, 2010; Benazir *et al*., 2011), groundnut cake (Kumar *et al*., 2008), varieties of rice broken (Paranthaman *et al.*, 2009), sunflower meal, soybean meal, cotton seed meal, rice polish (Ahmed *et al.*, 2010), coffee by-products (coffee pulp, coffee cherry husk, coffee parchment husk, silver skin, coffee spend wastes) (Murthy and Naidu, 2010), coconut bran, gingely oil bran, ground oil cake and black gram bran (Benazir *et al*., 2011). All the mentioned researches that utilized wastes as substrates in their studies discovered that although with the usage of cheap and easily available substrates but it really proved to efficiently produce enzymes.

The utilization of wastes in the fermentation of other several enzymes had also been reported. Gombert *et al.* (1999) has successfully produced lipase with the use of oil industrial waste (babassu oil cake) as substrate while Sidkey *et al.* (2010) able to utilize enviro-agro-industrial waste from food and drinks industry in the production of α-amylase. Ten different agroindustrial wastes were also reported in producing glucoamylase (Zambare, 2010).

According to Mahmood *et al.* (1998), the usage of agricultural wastes in developing countries are underutilized and if used but only in the production of single cell protein. On the contrary, in developed countries, these wastes are really useful in fermentation but still rarely used in enzymes production. The results from the studied recommended that these wastes can be used for better advantage. The common agricultural wastes are rice husk, coffee pulp and also potato peel.

According to Arapoglou *et al.* (2009) in the International Conference on Environmental Science and Technology entitled “Alternative Ways for Potato Industries Waste Utilization” in Greece, it was suggested to utilize wastes from the potato industries to be used for something beneficial since the problem due to this issue is of great concern. In order to overcome this problem, an environmental friendly solution is still under

investigation. In potato industry, potato starch waste and potato peel waste are the major wastes to be produced and about half of the potato industry waste production is monopolized by potato skin. In addition, potato peels contain sufficient amount of starch, cellulose, hemicellulose and fermentable sugars.

They have been used in the previous study and were approved good to serve as substrate in the production of extracellular protease enzymes (Mahmood *et al.*, 1998; Arapoglou *et al.*, 2009) and Table 2.4 shows the findings in a research that utilized potato and orange peel extracts as substrates.

Table 2.4: Effect of type of substrates on the production of extracellular proteases

Substrate	**Protease Activity (Units/ml)**	
	Alkaline protease	**Neutral protease**
Orange filtrate	7.5	20.0
Potato filtrate	11.0	33.0
Orange:Potato (1:1)	11.0	17.0
Glucose	4.0	7.5

Source: Mahmood *et al.* (1998)

From the table above, waste filtrates had been proved to potentially secrete optimum amount of protease compared to the utilization of glucose as substrate and potato peel extract presented the highest protease activity among the others. This issue is promising for industrial application (Arapoglou *et al.*, 2009).

2.7 OPTIMIZATION OF PROTEASE PRODUCTION

Nowadays, researchers are more focused in optimizing the process parameters so that high yield of protease can be achieved. Optimization is classically done by applying one-factor-at-a-time (OFAT) method where it is known to be very time-consuming method. There are several parameters are going to be optimized but in this method other parameters needs to be constant while studying a parameter at a time.

Fermentation is really affected by various factors such as pH, temperature, agitation speed, type of carbon and nitrogen sources and many others. In optimization, the best value for every studied factor is to be determined for its highest production of protease. For example, the effect of temperature to the fermentation process has been studied so that the exact temperature at which the protease yield is relatively high can be determined but by implementing OFAT method, each factor can only be studied alone since the other factors need to maintain constant. This method used to consume a long period of time if many parameters have to be studied. Furthermore, the effect of interaction between parameters cannot be observed in this way. The results obtained from OFAT can be further studied for the interaction between two or several factors.

Table 2.5 shows the optimization of parameters done by previous researcher. The research studied different parameters and came out with different outcomes (maximum protease activity). From the study, the researcher manage to identify at what range of parameters will actually result in high protease activity.

Unfortunately, the analysis for this kind of results is hard to be done. Therefore, Response Surface Methodology (RSM) is being introduced to facilitate in statistical analysis. In addition, RSM also helps in designing the experiment so that the experiment will cover all the range of parameters.

Table 2.5: Optimization of several parameters with the optimum protease activity

Species	State	Studied parameter	Value	Optimum protease activity
Aspergillus flavus	SmF	Incubation time	144 h	0.96 U/ml
		Temperature	30 °C	0.46 U/ml
		pH	8.0	0.74 U/ml
Aspergillus fumigatus		Incubation time	144 h	0.84 U/ml
		Temperature	30 °C	0.43 U/ml
		pH	5.0	0.70 U/ml

SmF submerged fermentation

Source: Oyeleke *et al.* (2010)

2.8 RESPONSE SURFACE METHODOLOGY

Optimization is important in order to improve the performance of the systems and to maximize the productivity of the process without increasing the cost (Bas and Boyaci, 2007). There will be many variables to be studied in every fermentation process. Studying one parameter at a time while holding the other parameters constant is the optimization process that is done classically and this will normally takes such a long period of time and quite costly, when large number of variables are evaluated (Saravanakumar *et al.*, 2010). This technique is called one-factor-at-a-time (Bas and Boyaci, 2007). It also does not consider the effect of interaction of various parameters (Adinarayana and Ellaiah, 2002; Bas and Boyaci, 2007) and it does not represent the complete effects of the parameters (Bas and Boyaci, 2007) so that is why Response Surface Methodology (RSM) is being introduced and recommended for optimization purpose.

Annadurai and Sheeja (1998) described RSM as an empirical modelization method used in the evaluation of the relationship of a set of controlled experimental factors and observed responses. RSM is a useful tool for studying factors that affect the responses by varying them simultaneously and it can also be used to study the relationships between one or more factors (independent variables) and responses (dependant variables) (Adinarayana

and Ellaiah, 2002). RSM can be applied in both chemical and biochemical processes (Bas and Boyaci, 2007).

Bas and Boyaci (2007) said that while using RSM, the optimization process should undergo three vital stages. The first stage is the preliminary study for estimating the independent parameters to be carry out. The second stage is choosing the experimental design and also predicting and verifying the model equation. The last stage is used to obtain the response surface plot and contour plot of the response and finally determining the optimum values.

The experimental design is required to allow the quadratic model to be fitted and minimized lack of fit of the model but still having enough degrees of freedom for pure error determination (Khuri, 2006). An efficient analysis should satisfy the following criteria. First, the second order model should efficiently fit. Second, the lack of fit of the model is also checked. Then, the unambiguous factors are selected and finally, the final model should also efficiently fit.

In addition, the interaction between the parameters can be clearly illustrated by response surface 3D plots (Dutta *et al.*, 2004) and also contour plots. RSM offers significant number of advantages compared to classical optimization method (Bas and Boyaci, 2007). First, RSM provides more information although with small number of experiments. Second, with RSM, the interaction between parameters can be study. In order to achieve optimization, RSM will reduce the number of trials (Gan *et al.*, 2007; Adinarayana and Ellaiah, 2002) and provide multiple regression approach (Gan *et al.*, 2007).

Besides, RSM also possess its negative part where the data needs to fit to second order polynomial but not all systems can be represented by second order polynomial (Bas and Boyaci, 2007). This problem can be eliminated by converting the data into other relevant form and can be explained by second order polynomial or if the second order

model make harder in explaining the system, narrowing the range of independent parameters.

Research by Bas and Boyaci (2007) also discovered that RSM cannot be used for optimization of all chemical and biochemical processes without any limitation and RSM also cannot be used in optimizing other purposes such as estimation of reaction kinetics. This is all because it is only usable for data that can be explained by second order model and not all systems can be fitted in second order model.

RSM is reported to be effective in optimizing various process parameters, levels of ingredients and formulation for variant products such as cassava cake (Gan *et al.,* 2007), adsorption of verofix red using biopolymer (Annadurai and Sheeja, 1998), purification of lipase (Gopinath *et al.*, 2003), extracellular protease production from *Pseudomonas* sp. (Dutta *et al.*, 2004) and extracellular alkaline protease production from *Aspergillus fischeri* (Saravanakumar *et al.*, 2010) have been reported by different researchers.